Further Observations on a Bacteriolytic Element found in Tissues and Secretions.

By Alexander Fleming, F.R.C.S., and V. D. Allison, M.B., B.Ch.

(Communicated by Sir Almroth Wright, F.R.S. Received June 16, 1922.)

(From the Department of Systematic Bacteriology, St. Mary's Hospital.)

[Plate 6.]

In a previous communication one of us* (A. F.) described an anti-bacterial substance (lysozyme) normally present in the tissues and secretions of the body. Further experiments have been done in connection with this substance, and some of the results obtained are here described.

Development of Strains of Bacteria Resistant to Lysozyme.

It was shown in the earlier communication that lysozyme existed in the tissues and secretions affecting many microbes, but that one type of coccus, which was called the *Micrococcus lysodeikticus*, was especially suitable for experimental purposes with this lytic substance as it was particularly susceptible to the lytic action, so that if a small quantity of lysozyme-containing material was embedded deep in the centre of an agar plate, and then the whole surface of the plate was thickly planted with this microbe, there was inhibition of growth of the coccus for a considerable area around the embedded tissue. It was found, however, that if these culture plates were kept for two or three weeks there developed in the zone of inhibition a few isolated colonies of a microbe which appeared to be identical with *M. lysodeikticus*. The appearance of such a plate is shown in fig. 1 (Plate 6).

It was thought that these colonies were produced from certain of the cocci which were especially resistant to the lysozyme of the particular tissue, and experiments were carried out to see whether the descendants of these "resistant" cocci were actually resistant to the lysozyme. Cultures were made from these isolated colonies which had developed close to the embedded material on culture plates, in which several different tissues and secretions had been embedded, and these cultures were labelled "resistant" to the particular tissue with which they had been grown in contact. Thus the term "tonsil resistant strain" means a culture derived from a colony which had been grown in close proximity to a piece of tonsil embedded in the agar. The action of lysozyme on these "resistant" strains, as compared with the

* Fleming, 'Roy. Soc. Proc.,' B, vol. 93 (1922).

ordinary stock culture of *M. lysodeikticus*, was first tested with the homologous tissue extract, *i.e.*, the "liver resistant strain" was tested with liver extract, the "tonsil resistant strain" was tested with tonsil extract, and so on, and the results are set forth in Table I.

Table I.—Comparison of Normal and "Resistant" Strains of *M. lysodeikticus* as regards their Solubility by the Homologous Tissue Extract or Secretion.

Fluid tested.	Strain of *M. lysodeikticus* used.	Dilution of fluid. 1/2	1/4	1/8	1/16	1/32	Control.
Liver extract	Non-resistant	+	+	+	AC	tr	0
	"Liver resistant"	±	±	0	0	0	0
Tonsil extract ...	Non-resistant	+	+	+	AC	±	0
	"Tonsil resistant"	±	0	0	0	0	0
Kidney extract ...	Non-resistant	+	+	+	AC	tr	0
	"Kidney resistant"	0	0	0	0	0	0
Intestine extract	Non-resistant	+	+	+	+	AC	0
	"Intestine resistant"	AC*	±	±	tr	0	0
Tears	Non-resistant	+	+	+	+	+	0
	"Tear resistant"	AC*	AC*	AC*	AC*	AC*	0
Nasal mucus	Non-resistant	+	+	+	+	+	0
	"Nasal mucus resistant"	AC*	AC*	AC*	AC*	±*	0

Time of incubation in tests, 15 minutes. Original dilution of fluid arbitrary, but in each experiment the same when testing the resistant and the non-resistant strain.

* These did not become absolutely clear even after many hours incubation.

+ = complete lysis. ± = partial lysis. AC = almost complete lysis. tr = trace of lysis.

From this Table it will be seen that in every case the "resistant" strain was much less affected than the normal strain by the lytic action of the homologous tissue or secretion. There were, of course, differences in the degree of resistance, and in no case was the resistance to lysis absolute. It was very noticeable, however, that with all the "resistant" strains there was never complete lysis of the cocci, as shown by an absolute clearing of the opaque suspension of the cocci. There was always a certain small amount of opacity left, which did not disappear even after many hours of incubation with the lytic material. This is especially shown in Table I in connection with tears and nasal mucus. In each of those cases the dilution chosen was so strong that after 15 minutes there was complete solution of the non-resistant culture in the highest dilution used; whereas, in the case of the corresponding "resistant" strains, the lysis was not quite complete in the strongest dilution of the lytic material, and the degree of lysis (almost complete) was the same in a number of the dilutions, which is never seen with an ordinary non-resistant strain of the coccus. It would appear, therefore, that in the "resistant" strains there are a considerable number of cocci which are absolutely resistant to lysozymic action, and this was further borne out by

planting these resistant strains on agar plates in which the homologous lysozyme-containing material had been embedded. In all cases, a fair number of colonies developed over or close to the embedded material, and in the case of tissues which were originally weak in lysozyme there was a continuous sheet of growth over the tissue.

Question as to whether a Strain of Microbe made Resistant to one Tissue is Resistant to other Tissues.

This was tested, in the first place, by taking organ extracts in various (arbitrary) dilutions, and adding to these a measured amount of suspensions of cocci derived from strains of *M. lysodeikticus* which had been made resistant to various organs and secretions, the control suspension being one of the normal cultures of the coccus. These tubes were incubated, and after 1 hour the amount of lysis was noted. The results obtained are given in Table II, and from this it will be seen that there is no indication of specificity in the resistance. Cocci grown in near proximity to one organ are not more resistant to the lytic action of an extract of that organ than they are to extracts of other organs.

Table II.—Comparison of "Non-resistant" and various "Resistant" Strains of *M. lysodeikticus* in regard to their Capacity of being Dissolved by Heterologous Lysozyme containing Fluids.

Coccus resistant to	Liver extract.	Tonsil extract.	Kidney extract.	Spleen extract.	Nasal mucus.	Intestine extract.	Control (Salt solution 0·85 per cent.).
Liver	0	+	tr	tr	?	±	0
Tonsil	0	tr	0	0	±		0
Kidney	0	tr	0	0	±	tr	0
Spleen	0	0	0	±	+	AC	0
Tears	0	tr	0	0	±	tr	0
Nasal mucus	0	0	0	0	±	0	0
Intestine	0	±	0	±	±	+	0
Control Non-resistant	±	AC	±	+	+	+	0

The same question was investigated in another manner by taking a strain of *M. lysodeikticus*, which had been made resistant to nasal mucus, and testing the lytic action of different organ extracts and secretions to this strain and to a normal non-resistant strain. This was done by making dilutions of the various lytic fluids and adding to one set of dilutions measured quantities of the "nasal mucus resistant strain," and to the other set the same quantities of the non-resistant strain. The tubes were then incubated at 50° C. for 1 hour and the amount of lysis noted. The results appear in Table III.

Table III.—Lytic Action of Various Secretions and Organ Extracts on a "Nasal Mucus Resistant" Strain of *M. lysodeikticus*.

	Non-resistant strain. Dilution.						Resistant strain. Dilution.					
	1/1	1/2	1/4	1/8	1/16	Control.	1/1	1/2	1/4	1/8	1/16	Control.
Nasal mucus (1 in 1000)	+	AC	±	tr	0	0	AC	? tr	0	0	0	0
Liver (1 in 400)	AC	±	±	0	0	0	±	0	0	0	0	0
Tonsil (1 in 200)	+	+	AC	±	±	0	±	±	0	0	0	0
Kidney (1 in 100)	+	AC	±	0	0	0	?	0	0	0	0	0
Tears (1 in 2000)	+	+	+	+	AC	0	AC	AC	±	±	0	0
Egg white (1 in 2000)	+	+	+	+	AC	0	AC	AC	±	±	0	0
Intestine (1 in 500)	+	AC	±	tr	0	0	tr	0	0	0	0	0
Stomach (1 in 1000)	+	AC	±	±	? tr	0	±	tr	tr	tr	0	0
Meninges (1 in 400)	AC	±	tr	0	0	0	tr	0	0	0	0	0
Skin (1 in 100)	±	±	?	0	0	0	0	0	0	0	0	0
Joint fluid (1 in 10)	+	+	+	+	AC	0	±	±	tr	tr	tr	0
Turnip (1 in 2)	+	+	AC	±	0	0	±	0	0	0	0	0

Note.—From Table III can be gathered some idea of the relative amount of lysozyme affecting *M. lysodeikticus* which is contained in the various organs, secretions, etc. It will be seen that after one hour's incubation there was complete lysis of the cocci in tears and egg-white diluted 1 in 16,000, whereas extract of turnip only gave complete lysis in a 1 in 4 dilution.

It will be seen from this Table that the resistance to lysis of the "nasal mucus resistant strain" was approximately the same to all the materials tested, whether liver, joint fluid, egg white, turnip, etc. This would seem to show, therefore, that the lysozyme (affecting the *M. lysodeikticus*) contained in such widely different substances as liver, tears, egg white, and turnip is essentially the same.

The non-specificity of the lysozyme contained in different organs and secretions, can be demonstrated also by embedding in a streak across an agar plate some lysozyme-containing material and then stroking across this different strains of *M. lysodeikticus*. Fig. 2 illustrates such a plate where tears were embedded in the agar, and across the streak in which the tears were embedded we stroked a non-resistant strain of *M. lysodeikticus*, and strains which had been made resistant to various tissues and secretions. It will be seen that with all the "resistant" strains the area of inhibition is much less than it is with the non-resistant strain, and it will also be seen that the strain which had originally been made resistant to tears was not more resistant to the tear lysozyme than were the other "resistant" strains.

Influence of Solution of M. Lysodeikticus *on the Lysozyme-content of the Lytic Fluid.*

In bacteriology we are confronted with two opposite results of the action of a biological bacteriolytic agent. In the case of the bacteriolytic agent in

blood serum, which may be developed as the result of immunising an animal, it has long been known that this is wholly removed from the serum by saturation with the microbe on which the bactericidal agent acts. In this case there seems to be a combination between the bactericidal agent and the bacteria, with the result that the former is wholly fixed. On the other hand, we have the phenomenon of the "bacteriophage," where the admixture of a small quantity of the lytic agent with a young culture of the microbe leads to the production of a very large amount of the lytic substance.

Experiments were done with a view to determining whether, in the process of solution of the *M. lysodeikticus* by the lysozymic action of secretions, the lytic substance was destroyed like the immune bacteriolytic agent of the blood serum, or whether it was increased as is the "bacteriophage."

The first experiment was designed to ascertain whether, after a lysozyme-containing fluid had completed the solution of large numbers of *M. lysodeikticus*, the resultant fluid still possessed lytic properties towards this microbe.

Forty c.mm. of tears (1 in 100) were mixed with the same volnme of a very thick suspension of *M. lysodeikticus* and incubated for 5 minutes at 50° C., when lysis of the cocci was complete. Meanwhile, the same volume of the same dilution of tears was mixed with 40 c.mm. of normal salt solution. Each of these two volumes of 40 c.mm. of 1 per cent. tears were then blown out of the pipettes into 2 c.c. of salt solution and thoroughly mixed, making a dilution of the tears of 1 in 5000.

These solutions were then titrated for lytic substance by making a series of two-fold dilutions, adding one drop of a thick suspension of *M. lysodeikticus*, so that the final opacity was suitable for the observance of lysis, and incubating for 1 hour at 50° C. The results obtained were as follows:—

Tears + salt solution.		Tears + *M. lysodeikticus*.	
Dilution 1 in—		Dilution 1 in—	
10,000	+	10,000	+
20,000	AC	20,000	AC
40,000	±	40,000	AC
80,000	0	80,000	±
160,000	0	160,000	±

This shows that in the 5 minutes during which complete lysis of the cocci took place there had been an increase in the content of lytic substance such that, whereas the original tears showed in 1 hour no lysis in a dilution of 1 in 80,000, the tears which had dissolved the cocci showed considerable lysis even in a dilution of 1 in 160,000.

The next experiment aimed at showing whether the amount of the increase in lytic substance was dependent on the number of microbes which had been dissolved.

Here a 1 in 100 solution of tears was employed, and to measured quantities of this solution graded quantities of *M. lysodeikticus* were added, and the mixtures were incubated at 50° C. for 15 minutes when lysis was complete. The fluids, as well as two control tubes in which salt solution had been substituted for the suspension of cocci, were then titrated for lytic power. The results are given in Table IV, and it will be seen that the increase in lytic power is dependent on the number of cocci which had been dissolved. It would appear, therefore, that in the process of lysis of the bacteria a substance is produced which can exert a bacteriolytic action.

Table IV.—Showing the Increase in the Lytic Power of Tears after Solution of varying Quantities of *M. lysodeikticus*.

Number of cocci on which tears had acted and dissolved.	*M. lysodeikticus.* Dilution (thousands)							Intestinal streptococcus. Dilution (hundreds).						
	5	10	20	40	80	160	320	1	2	4	8	16	32	64
0 (control)	+	AC	±	0	0	0	0	+	+	AC	±	±	tr	0
0 (control)	+	AC	±	0	0	0	0	+	+	AC	±	±	tr	0
30 per c.c.	+	AC	±	tr	0	0	0	+	+	AC	±	±	tr	0
300 ,, ...	+	+	AC	AC	±	0	0	+	+	AC	±	±	tr	0
3,000 ,, ...	+	+	+	AC	±	0	0	+	+	AC	±	±	tr	0
30,000 ,, ...	+	+	+	AC	±	tr	0	*	*	AC	±	±	tr	0
	Tests incubated 1 hour at 45° C.							Tests incubated 2 hours at 45° C.						

* The fluid in these tubes was clouded from the *débris* resulting from the solution of the large number of cocci which had been acted on, so that accurate readings could not be made.

This increase in the lytic power is maintained if second and third additions of large amounts of cocci are added and dissolved. This is shown in Table V, which gives the results of titration of lytic substance in tears, 1 in 100, to which had been added, on three successive days, an equal volume of a suspension of *M. lysodeikticus*, containing 30,000 million cocci per c.c. These are contrasted with the control tubes which had normal salt solution

Table V.—Showing the Increase in the Bacteriolytic Power of Tears after Successive Additions and Lysis of Large Numbers of *M. lysodeikticus*.

Fluid tested.		Dilution of tears (thousands).							
		5	10	20	40	80	160	320	640
Control.....	1st day ...	+	AC	±	0	0	0	0	0
Tears which had not dissolved any cocci	2nd ,, ...	+	+	±	?	0	0	0	0
	3rd ,, ...	+	+	±	tr	0	0	0	0
Tears which had dissolved 30,000 million cocci per c.c. on each of three successive days	1st day ...	+	+	+	AC	±	tr	0	0
	2nd ,, ...	+	+	+	+	AC	±	tr	0
	3rd ,, ...	+	+	+	+	AC	±	tr	tr

substituted for the coccal suspension, but which otherwise went through the same procedures. It will be seen that there is a progressive increase in the lytic power with the addition of a fresh amount of the thick coccal suspension, although the power of the control tubes remains practically stationary.

The increase in the lytic power after solution of large numbers of cocci can also be shown graphically by embedding in an agar plate a measured volume of a lysozyme-containing fluid, and the same volume of the same fluid which has dissolved a large number of cocci. Fig. 3 represents such a plate, in which two volumes of nasal mucus were embedded, and also two similar volumes of the same nasal mucus which had dissolved a very large number of *M. lysodeikticus*. This organism was then thickly planted over the whole surface, and it will be seen that the area of inhibition in each case is greater where the nasal mucus had been previously allowed to act on the microbes.

Question as to whether this Increase in the Lytic Power is a General Increase affecting all Microbes, or whether it is an Increase Specific to the Microbe which has undergone Lysis.

Table IV shows that after solution of varying amounts of *M. lysodeikticus* there has been a very definite increase in the lytic power of tears to this microbe. When, however, the lytic power of these same fluids was tested to an intestinal streptococcus, it was found that there had been no increase in their power of dissolving the cocci. The results obtained are shown in Table IV, Column 2.

This specificity in the increase of lytic power to *M. lysodeikticus*, after solution of this microbe, is further brought out in another experiment in which egg white (1 per cent. in normal saline solution) was allowed to dissolve a thick suspension of *M. lysodeikticus*, and then the resulting fluid was tested for lytic power to this microbe, and to two other air-borne bacteria which were found to be specially sensitive to the lytic action of egg white. The results are shown on Table VI, and it will be seen that, whereas there is an increase in the lytic power to *M. lysodeikticus*, there is no increase to the other two microbes.

In other experiments of the same nature it was found that, after solution of *M. lysodeikticus*, there was an increase in the lytic power, not only to the stock strain of this microbe but also to certain other cocci which appeared to be identical, microscopically, with this, although in their chromogenetic properties there were certain differences, notably in one culture which developed a rich orange-brown colour in marked contrast to the bright lemon yellow of *M. lysodeikticus*. It is possible that these cocci were really of the same type in spite of the colour difference.

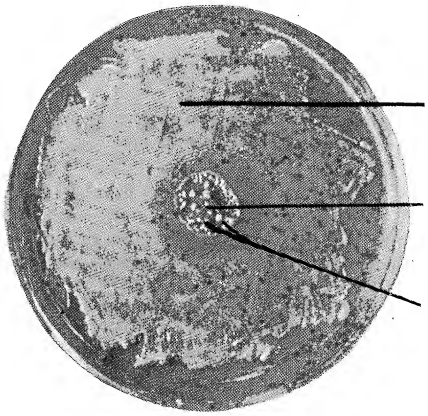
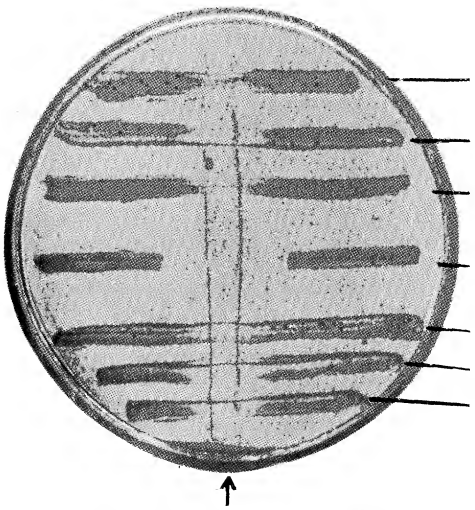


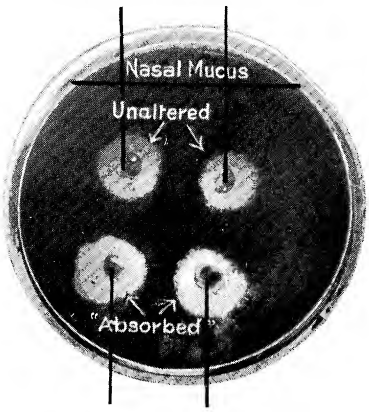
FIG. 1.—Development of "resistant" colonies of *M. lysodeikticus*.



Tears imbedded in the agar in a streak extending from the top to the bottom of the plate.

FIG. 2.—Illustrating the non-specificity of the "resistance" developed by growth of the cocci in close proximity to a tissue.

Unaltered nasal mucus imbedded.



Nasal mucus imbedded which had dissolved a large number of *M. lysodeikticus*.

FIG. 3.—Illustrating the increase in lytic power of nasal mucus after solution of a large number of *M. lysodeikticus*.

Table VI.—Showing the Specificity of the Increase in Lytic Power of Egg-White to *M. lysodeikticus* after the Egg-White has Dissolved a Large Number of this Type of Coccus.

Bacterium to which lytic power tested.	Unaltered egg-white. Dilution.						Egg-white which had dissolved a large number of *M. lysodeikticus*. Dilution.					
	1/10	1/20	1/40	1/80	1/160	1/320	1/10	1/20	1/40	1/80	1/160	1/320
M. lysodeikticus	+	+	+	AC	±	0	+	+	+	+	AC	±
Coccus A	+	AC	±	±	0	0	+	AC	±	±	0	0
„ B	+	+	±	±	±	±	+	+	±	±	±	±

It may be of interest, at this stage, to compare lysozyme with other bactericidal and bacteriolytic substances which have been described from time to time as having been found in the secretions and tissues. The work, prior to 1900, is considered in detail by Metchnikoff in his treatise on Immunity, and he sums it up as follows :—

"Nature does not make use of antiseptics to protect the skin and mucous membrane. The fluids which moisten the surface of the mouth and of other mucous membranes is not microbicidal, or only so to a very slight degree, and then of rather an exceptional nature. Nature rids the mucous membranes and the skin of a large number of micro-organisms eliminating them by epithelial desquamation, and expelling them along with fluid secretions and excretions. Nature, like the doctors of the present day who replace antisepsis of the mouth, intestine, and other organs, by washing with pure physiological saline solution, has chosen this mechanical method."

Since 1900, other publications have been made on this subject. Conradi* showed that from autolysed organs and tissues there could be extracted a bactericidal substance. He does not deal with bacteriolysis, and the properties of the substance which he describes are so widely different from those which we have shown are possessed by lysozyme, that it makes it quite clear that he was dealing with a different substance. Conradi's bactericidal substance was not destroyed by 4 or 5 hours boiling, it was easily filtered through clay candles, it was not adsorbed by animal charcoal, it was soluble in alcohol, and the reaction of the fluid made no difference to its action, in all of which respects it is entirely different from lysozyme.

Meissner† showed that from leucocytes bactericidal substances could be extracted, and, in connection with tears, he showed that, whatever

* 'Beiträge für Chemische Physiologie und Pathologie,' vol. 1 (1902).

† 'Zeitschrift für Hygiene und Infektionskrankheiten,' vol. 72 (1912).

bactericidal substance he obtained, was due to the cells which escaped into the fluid from the conjunctiva when this membrane was acted on by an irritant. He insists that normal tears (and by these we presume he means tears evoked by emotion) have no bactericidal properties. We have demonstrated that the lysozyme-content of emotional tears is exactly the same as that of tears produced by a mild irritant. Meissner, also, makes no mention of bacteriolysis, which is an essential property of lysozyme.

We have mentioned that egg-white contained a large amount of lysozyme, and we have used it in some of our experiments. It had previously been shown by Laschtschenko,* and following him by Rettger and Sperry,† that egg-white possessed bactericidal properties, but again no mention is made of bacteriolysins.

Gengou‡ extracted from leucocytes a bacteriolytic substance, which has some properties resembling those of lysozyme, but which differs materially in certain characters, notably in its being inhibited by inactivated serum or ascitic fluid (which themselves contain lysozyme), and in its being completely absorbed by saturation with any of the bacteria that it is capable of dissolving, whereas we have shown that with lysozyme there is an increase in the lytic substance after solution of very large quantities of *M. lysodeikticus*.

Turro§ has shown that extracts of leucocytes and many other tissues have a strong bacteriolytic action on *B. anthracis* and other bacteria. He does not enter into sufficient detail regarding the properties of this lysin to make it clear whether it is of the same order as the lysozyme which we have described. In one of the few properties which he mentions, however, there seems to be a great difference. He states, in connection with his work on leucocytes, that the bacteriolytic power is rapidly lost, either in the saline extract or in the dry powder, so that they may be inert after three to five days. We have found that lysozyme is very stable, and does not apparently deteriorate with keeping, for instance, we have found that dried commercial egg albumen is very rich in lysozyme, although it has probably been kept in the dried form for many months or even years.

Arising out of Turro's work is the work of Kuttner,‖ who succeeded in extracting from various organs and tissues transmissible lytic substances with properties similar to the lytic agent described by Twort, d'Herelle, and

* 'Zeitschrift für Hygiene,' vol. 64 (1909).
† 'Journal of Medical Research,' vol. 26 (1912).
‡ 'Annales Institut Pasteur,' vol. 35 (1921).
§ 'C. R. Soc. Biologie,' vol. 84 (1921).
‖ 'Proc. Experimental Biology and Medicine,' vol. 18 (1921).

later by many other authors, and which has come to be known as the "Bacteriophage." It is unnecessary here to go into the theories as to the nature of the "Bacteriophage" and its exact relationship to the lytic substance we have described (lysozyme). The extreme rapidity of its action, the wide range of temperature through which it will act, the fact that it will dissolve dead as well as living bacteria, the difficulties experienced in passing small quantities through a porcelain filter, and the absence in solid cultures of the small clear areas of lysis, mark out lysozyme as something different from the bacteriophage.

Summary.

Strains of *M. lysodeikticus* resistant to lysozyme action can readily be developed.

The resistance is not specific, *i.e.*, strains made resistant to one tissue or secretion are equally resistant to all tissues, whether derived from man, the lower animals, or from vegetables, showing that the lysozyme affecting *M. lysodeikticus* is the same whatever is the tissue it is derived from.

After solution of a large number of *M. lysodeikticus* there is an increase in the lytic power of the fluid, and this increase affects wholly or mainly the homologous microbe.

The Pigmentary Effector System.—II.

By Lancelot T. Hogben and Frank R. Winton.

(Communicated by Prof. E. W. MacBride, LL.D., F.R.S. Received August 1, 1922.)

(From the Zoological Laboratory, Imperial College of Science.)

CONTENTS.

1. *Introduction.*

In a previous paper dealing with the pigmentary response evoked by pituitary (posterior lobe) administration in the common frog, emphasis has been laid on the necessity of discriminating between the alternatives of nervous and

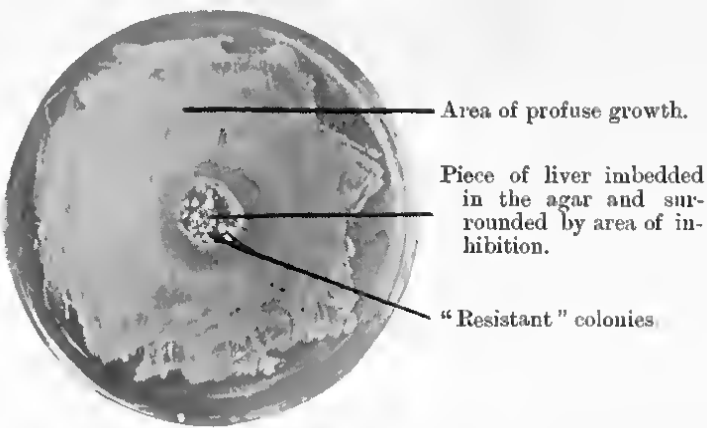


FIG. 1.—Development of "resistant" colonies of *M. lysodeikticus*.

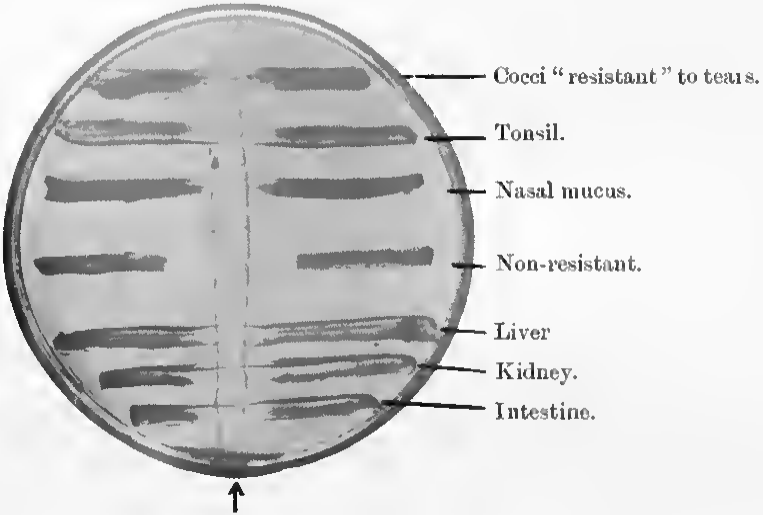
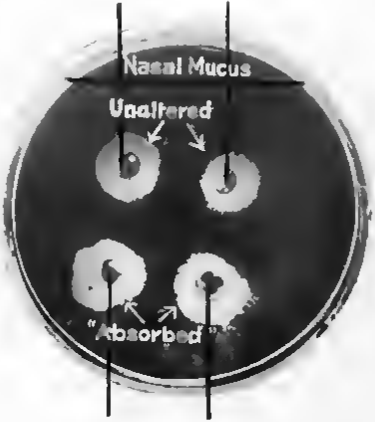


FIG. 2.—Illustrating the non-specificity of the "resistance" developed by growth of the cocci in close proximity to a tissue.

Unaltered nasal mucus imbedded.



Nasal mucus imbedded which had dissolved
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FIG. 3.—Illustrating the increase in lytic power of nasal mucus after solution of a large number of *M. lysodeikticus*.